MicroRNA-195-5p regulates carbon monoxide releasing molecule-3-induced osteogenic differentiation of rat bone marrow mesenchymal stem cells by targeting Wnt3a

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Abstract

Background and Purpose: Bone marrow-derived mesenchymal stem cells (BMSCs) are potential in promoting bone regeneration for their multipotential differentiation capacity. Our previous study showed that carbon monoxide releasing molecule-3 (CORM-3) promoted the osteogenic differentiation of rat BMSCs. However, the mechanism was not clearly understood. MicroRNAs (miRNAs) play a critical role in regulating the osteogenic differentiation of BMSCs. We therefore investigated the role of miR-195-5p in CORM-3-induced osteogenic differentiation. Experimental Approach: The rat BMSCs were transfected with miR-195-5p mimics, miR-195-5p inhibitor, pcDNA3.1-Wnt3a, Wnt3a siRNA or their corresponding controls. The rat BMSCs osteogenic differentiation was assessed by quantitative real-time polymerase chain reaction, Western blot and alizarin red staining. In addition, dual luciferase assay was used for the verification of targeting relationship between miR-195-5p and Wnt3a. Key Results: miR-195-5p was down-regulated during the CORM-3-induced osteogenic differentiation of rat BMSCs. Overexpression of miR-195-5p inhibited CORM-3-induced osteogenic differentiation of rat BMSCs, evidenced by significantly decreased mRNA and protein expressions of runt-related transcription factor 2 and osteopontin, and matrix mineralization demonstrated. Whereas, inhibition of miR-195-5p expression enhanced osteogenic differentiation. miR-195-5p directly targeted Wnt3a. Overexpression of Wnt3a increased CORM-3-induced osteogenic differentiation of rat BMSCs, the opposite effect was observed in Wnt3a-deficient cells. Moreover, the inhibitory effect of miR-195-5p overexpression on CORM-3-induced osteogenic differentiation was rescued by Wnt3a overexpression. Conclusion and Implications: These results demonstrated that miR-195-5p may negatively regulate CORM-3-induced osteogenic differentiation of rat BMSCs by targeting Wnt3a, which provided insight into new mechanism of CORM-3, and theoretical basis for bone regeneration.

Introduction

Reconstruction and healing of bony defects remains a major clinical challenge. Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent cells that can differentiate into a variety of cell types characteristic of bone, skeletal and cardiac muscle, adipose tissue, and neural cells (Dai *et al.* , 2018; Jing*et al.* , 2019; Luby *et al.* , 2019). Numerous studies have demonstrated that BMSCs are potential in promoting bone regeneration and defect repair (Luby *et al.* , 2019; Polymeri *et al.* , 2016).

MicroRNAs (miRNAs) are non-coding RNAs that are approximately 22 nucleotides in length. miRNAs play important roles in post-transcriptional regulation by interacting with the 3' untranslated region (3'UTR) of target genes, which induces mRNA degradation or suppresses protein translation (Bartel, 2004; Mohr *et*

al. , 2015; O'Brien *et al.* , 2018). A series of miRNAs have been characterized as regulators of osteogenic activity and osteoblastic bone formation, either positively or negatively, through multiple signaling pathways (Hata *et al.* , 2015; Vimalraj *et al.* , 2013; Zhang *et al.* , 2017). miRNAs plays a critical role in regulating the osteogenic differentiation of BMSCs (Li, 2018; Wang *et al.* , 2019). For example, miR-149-3p regulates the switch between adipogenic and osteogenic differentiation of BMSCs by targeting FTO (Li *et al.* , 2019). Moreover, miR-488 is a negative regulator of psoralen-induced osteogenic differentiation of BMSCs by targeting Runx2 (Huang *et al.* , 2019).

Carbon monoxide releasing molecules (CORMs) are newly identified transition metal carbonyl-based compounds, able to efficiently regulate the release of CO (Motterlini *et al.* , 2003). Previously, we have discovered that CORM-3 promoted the osteogenic differentiation of rat BMSCs (Li *et al.* , 2018), and the same result was acquired in the periodontal ligament cells (PDLCs) (unpublished data). However, the underlying molecular mechanism remains unclear. In the experiments of our research group, the gene sequencing result of CORM-3-stimulated PDLCs showed that miR-195-5p was significantly down-regulated during osteogenic differentiation. To date, the role of miRNAs in CORM-3-induced osteogenic differentiation is poorly understood. As miRNAs are highly conserved, we hypothesized that miR-195-5p might be a regulator of CORM-3-induced osteogenic differentiation of rat BMSCs. In the present study, the effects of miR-195-5p on CORM-3-induced osteogenic differentiation of rat BMSCs, and its regulatory pathway were investigated *in vitro,* which was aimed to provide insight into the mechanism underlying the beneficial effects of CORM-3 and to explore the new potential therapy approach for bone regeneration.

Methods

Cell culture and identification

4-5 weeks old male Wistar rats were obtained from the Animal Experimental center of Shandong University (Jinan, china). The present study was approved by the Ethics committee of the School of Stomatology, Shandong University. Rat BMSCs were isolated and cultured as previously described (Li *et al.* , 2018). Briefly, after rats were euthanized, the femur and tibia were removed and the bone marrow cavity was rinsed with α-minimum essential medium (α-MEM) supplemented with 20% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. Bone marrow fluid was incubated at 37@C in an atmosphere containing 5% CO_2. The medium was changed every 3 days, non-adherent cells were discarded. When cells reached 80-90% confluence, they were sub-cultured in α-MEM supplemented with 10% FBS (control medium). Rat BMSCs of passage 3 were used in the following experiments.

For osteogenic induction, cells were cultured in the osteogenic medium (osteogenic group). Cells in control group were cultured in the control medium. Following 21 days culture, cells were fixed with 4% paraformaldehyde at 37@C for 30 min, then incubated with 0.1% (pH 4.2) alizarin red S at 37@C for 10 min. After washing with phosphate buffered saline (PBS), samples were observed using the phase-contrast microscope to verify the presence of mineralized nodules.

For adipogenic induction, cells were cultured in the adipogenic medium as previously described (Li *et al.* , 2018). Cells in control group were cultured in the control medium. Following 21 days culture, cells were fixed as mentioned above. Then cells were incubated with oil red O at 37@C for 30 min and observed using the phase-contrast microscopy.

For clonogenesis experiment, rat BMSCs were seeded at a density of 3 cells/cm^2 and cultured in control medium. Following 10 days culture, cells were fixed with 4% paraformaldehyde for 30 min, then stained with 0.1% crystal violet for 10 min at 37@C. After washing with the tri-distilled water, cell clone was observed using the phase-contrast microscope. More than 50 cells were regarded as one cell clone. The clone formation rate was calculated as the number of cell clone / the total number of seeded cells × 100%, and the average value of six samples was obtained.

For subsequent CORM-3-induced osteogenic differentiation experiments, cells were cultured in the osteogenic medium containing 200 μM CORM-3 (CORM-3 group).

Cell viability assay

BMSCs were seeded in 96-well plates at a density of 5x10^3 cells/cm^2 and cultured in control medium for 24 hours at 37@C. Subsequently the medium was removed and cells were cultured in fresh control medium containing 0 (control group), 100, 200 and 400 μM CORM-3. At 24, 48 and 72 hours, 10 μl cell counting Kit-8 was added to each well and cells were incubated for a further 2 hours at 37@C. Subsequently, absorbance at 450 nm was measured using SPECTROstar Nano ultraviolet spectrophotometer (Spectro Analytical Instruments GmbH, Kleve, Germany). The experiment was done in triplicate, and repeated for three times.

Transfection

The rat BMSCs were transfected with miR-195-5p mimics, miR-195-5p inhibitor, pcDNA3.1-Wnt3a, Wnt3a siRNA or their corresponding controls using Micropoly-transfecter Cell Reagent according to the manufacturer's instructions. 24 hours after transfection, the medium was replaced with control medium. 48 hours after transfection, cells were harvested for miR-195-5p and Wnt3a measurement. For subsequent osteogenic differentiation experiments, after 24 hours transfection, the medium was completely replaced with the osteogenic medium containing 200 μM CORM-3. In rescue experiment, cells were co-transfected with miR-195-5p mimics and pcDNA3.1-Wnt3a, miR-195-5p mimics and vector NC. For miR-195-5p target gene experiment, after 24 hours transfection, the medium was completely replaced with control medium.

Quantitative real-time PCR analysis

The level of miR-195-5p after transfection and mRNA expressions of osteogenic-related genes runt-related transcription factor 2 (Runx2) and osteopontin (OPN) during osteogenic differentiation were evaluated by real-time quantitative reverse transcription PCR (RT-qPCR) as previously described (Li *et al.* , 2018). The primer sequences used were as follows: Runx2, forward 5'-CAGACACAATCCTCCCCACC-3', and reverse 5'-GCCAGAGGCAGAAGTCAGAG-3'; OPN, forward 5'-TCAAGGTCATCCCAGTTGCC-3', and reverse 5'-GACTCATGGCTGGTCTTCCC-3'; β-actin, forward 5'-CTCTGTGTGGATTGGTGGCT-3', and reverse 5'-CGCAGCTCAGTAACAGTCCG-3'; miRNA-195-5p, forward 5'- CGTTATCCTAGCAGCACAGAAAT-3', and reverse 5'-TATGGTTTTGACGACTGTGTGAT-3'; and U6, forward 5'-CAGCACATATACTAAAATTGGAACG-3', and reverse 5'-ACGAATTTGCGTGTCATCC-3'. Each sample was tested in triplicate and the relative gene expressions were calculated using the $2^{-\Delta\Delta^{\sim}T}$ method, with β-actin or U6 for normalization.

Western blot analysis

Protein lysates were generated with radio immunoprecipitation assay lysis buffer. Then protein concentrations were determined using BCA protein assay kit according to the manufacturer's protocol. Protein samples (20 μg/lane) were loaded onto 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electrotransferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat milk, membranes were incubated overnight at 4@C with primary antibodies, including rabbit anti-rat Runx2 monoclonal antibody (1:1,000 dilution), rabbit anti-rat OPN polyclonal antibody (1:1,000 dilution), and rabbit anti-rat Wnt3a polyclonal antibody (1:400 dilution). Then, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000 dilution) for 1 h at room temperature. Membranes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech; Little Chalfont, U.K.). Loading differences were normalized using rabbit anti-rat GAPDH monoclonal antibody (1:2,000 dilution) or rabbit anti-rat Tubulin polyclonal antibody (1:1,000 dilution). Protein band densities on scanned films were quantified using ImageJ 1.48u software (National Institutes of Health, Bethesda, Md, USA) and compared with the control.

Analysis of mineralization

Following 14 days culture, cells were fixed, then incubated with alizarin red S as mentioned above. For further evaluation, staining was dissolved in 100 μM cetylpyridinium chloride (CPC) for 1 h at 37@C. The

optical density (OD) value of the staining dissolved CPC was measured at 562 nm using the ELISA plate reader. All experiments were repeated for three times, and each experiment in triplicate.

Dual-luciferase reporter assay

A luciferase reporter assay was carried out using a Dual-Luciferase Reporter Assay System (Promega, WI, U.S.A.). A fragment of the Wnt3a 3'UTR containing the predicted binding site for miR-195-5p or the respective binding site of the mutant-type (mut) 3'UTR was inserted into the pmirGLO vector. All the constructs were verified by DNA sequencing. The vector containing wild-type (wt) or mut was transfected into the 293T cells with or without the miR-195-5p mimics. 24-48 hours after transfection, the luciferase activity was detected using the Dual-Luciferase Reporter Assay System and normalized to Renilla activity.

Statistical analysis

All experiments were repeated three times. Data were presented as the mean ± standard deviation. The significance of difference was assessed by one-way analysis of variance method using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA), followed by the Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Materials

Reagents were obtained from the sources as indicated: α-MEM (Hyclone, GE Healthcare Life Sciences, UT, USA), FBS, penicillin-streptomycin (Gibco, Invitrogen, CA, USA), osteogenic medium (Cyagen, Guangdong, China), alizarin red S, PBS, oil red O, crystal violet, radio immunoprecipitation assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), CORM-3 (Sigma-Aldrich, Shanghai, China), cell counting Kit-8 (Dojindo Molecular Technologies, Inc., Beijing, China), miR-195-5p mimics, miR-195-5p inhibitor, Wnt3a siRNA (GenePharma, Shanghai, China), pcDNA3.1-Wnt3a (BioSune, Shanghai, China), Micropoly-transfecter Cell Reagent (Micropoly, Jiangsu, China), BCA protein assay kit, SDS-PAGE gel (Boster Biological Technology Co., Ltd., Wuhan, China), PVDF membrane (Pall Corporation, Port Washington, NY, USA), anti-Runx2 antibody (cat no., 12556s, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-OPN antibody (cat no., ab8448, Abcam, Cambridge, Britain), anti-Wnt3a antibody (cat no., WL0199a, Wanleibio, Shenyang, China), secondary antibody (cat no., SA00001-2, Proteintech Group, Inc., Rosemont, IL, USA), anti-GAPDH antibody (cat no., CW0100, Beijin ComWin Biotech Co., Ltd., Beijing, China), anti-Tubulin antibody (cat no., 11224-1-AP, Proteintech), pmirGLO vector (Zorin, Shanghai, China).

Results

Identification of rat BMSCs and cell viability assay

Adherent cells were observed in culture dish after 3 days of incubation. Cells grew in colonies, and reached 80% confluence on the 7th day. These cells were designated as P0 cells. The P3 cells were uniform in shape, fusiform mainly, and grew in whirlpool shape (Fig. 1A). As indicated in Fig. 1B, at 24 hours, 100 and 200 μM CORM-3 significantly enhanced the cell viability compared with the control ($P < 0.05$), whereas 400 μM CORM-3 had no significant effect on the cell viability compared with the control ($P > 0.05$). No significant difference of cell viability was found between any two groups on 48 and 72 hours ($P > 0.05$). Cells were induced to differentiate into osteoblasts or adipocytes. The mineralized nodules was visualized by alizarin red staining (Fig. 1C) and lipid droplets were identified by oil red O staining (Fig. 1E). Control groups were negative for alizarin red (Fig. 1D) and oil red O staining (Fig. 1F). Cells were seeded at low density and cultured for 10 days. After crystal violet staining, cell clone was seen by the phase-contrast microscope, and the clone formation rate was (24.3 ± 2.78) % (Fig. 1G-H).

Expression of miR-195-5p and wnt3a in the CORM-3-induced osteogenic differentiation of rat BMSCs

As indicated in Fig. 2A, miR-195-5p level in cells cultured in COMR-3 group was obviously decreased compared with osteogenic group and control group on 24 hours ($P < 0.05$). The protein expression of wnt3a

was enhanced during the CORM-3-induced osteogenic differentiation of rat BMSCs (Fig. 2B). At 24, 48 and 72 hours, the protein expressions of wnt3a in COMR-3 group were increased by 3.3, 1.5 and 1.3-fold compared with osteogenic group, respectively ($P < 0.05$).

Effects of miR-195-5p on the CORM-3-induced osteogenic differentiation of rat BMSCs

A successful transfection with miR-195-5p mimics, or miR-195-5p inhibitor was confirmed by RT- qPCR (Fig. 3A). mRNA expressions of the Runx2 and OPN were assessed by RT-qPCR at different time points during osteogenic differentiation. After the overexpression of miR-195-5p, mRNA levels of both Runx2 and OPN were significantly lower than that in the CORM-3 group on the 3rd and 7th day of osteogenic induction ($P< 0.05$) (Fig. 3B) . While miR-195-5p was down-regulated, the mRNA expressions of Runx2 and OPN were increased by 1.4- and 1.5-fold, respectively, on the 3rd day ($P < 0.05$)), and both 1.3-fold on the 7th day of osteogenic induction, compared with CORM-3 group ($P < 0.05$) (Fig. 3C). In consistence with the regulatory effect of miR-195-5p on the mRNA expression of Runx2 and OPN, the protein expressions of Runx2 and OPN after the osteogenic induction were significantly decreased in miR-195-5p overexpressed cells, but increased in miR-195-5p deficient cells ($P < 0.05$) (Fig. 3D-E). The results from the alizarin red staining and semi quantitative analysis demonstrated that mineralization on 14 days of osteogenic induction after miR-195-5p mimics transfection was decreased obviously ($P < 0.05$), however, the opposite effect was not observed in miR-195-5p-deficient cells (Fig. 3F).

miR-195-5p directly targets Wnt3a

TargetScan, miRanda and miRBase were used to predict the potential targets of miR-195-5p. Among the candidate target genes, Wnt3a had a miR-195-5p-binding site in their 3'UTR (Fig. 4A). After up-regulation or down-regulation of miR-195-5p level in cells, the protein expression of Wnt3a was decreased or increased correspondingly ($P < 0.05$) (Fig. 4B-C). In order to test whether miR-195-5p directly targets Wnt3a, dual luciferase reporter assay was constructed, which had either the Wnt3a 3'UTR-wt or the Wnt3a 3'UTR-mut (Fig. 4A). The miR-195-5p mimics inhibited the luciferase reporter activity of the Wnt3a 3'UTR-wt by 19% ($P < 0.05$), however, the activity was markedly increased with the Wnt3a 3'UTR-mut and miR-195-5p mimics co-transfection ($P < 0.05$) (Fig. 4D).

Effects of Wnt3a on the CORM-3-induced osteogenic differentiation of rat BMSCs

A successful transfection with pcDNA3.1-Wnt3a or Wnt3a siRNA was confirmed by Western blotting (Fig. 5A) analysis. With overexpression of Wnt3a, the mRNA levels of Runx2 and OPN were increased by 1.34- and 1.82-fold respectively on the 3rd day ($P < 0.05$), and 1.40- and 1.29-fold respectively on the 7th day of osteogenic induction, compared with CORM-3 group ($P < 0.05$) (Fig. 5B). On the contrary, in Wnt3a-deficient cells, mRNA expressions of Runx2 and OPN were all reduced on the 3rd and the 7th day of osteogenic induction ($P < 0.05$) (Fig. 5C). The protein expressions of Runx2 and OPN after the osteogenic induction were similarly regulated by Wnt3a, in line with the regulation of Wnt3a on the mRNA expression (Fig. 5D-E). In Wnt3a-overexpression cells, the protein expressions of Runx2 and OPN were increased by 1.60- and 1.85-fold respectively on the 3rd day, 2.02- and 2.56-fold respectively on the 7th day of osteogenic induction, compared with CORM-3 group ($P < 0.05$). Moreover, mineralization in Wnt3a-overexpression cells on the 14th day of osteogenic induction was enhanced significantly, compared with CORM-3 group. The opposite effect was observed in Wnt3a-deficient cells ($P < 0.05$) (Fig. 5F).

miR-195-5p regulated CORM-3-induced osteoblastic differentiation of rat BMSCs by targeting Wnt3a

To verify whether the effect of miR-195-5p during CORM-3-induced osteoblastic differentiation depended on Wnt3a, rat BMSCs were co-transfected with miR-195-5p mimics and pcDNA3.1-Wnt3a or vector NC. With miR-195-5p up-regulated, the mRNA and protein expressions of Runx2 and OPN were increased after Wnt3a overexpression on the 3rd and the 7th day of osteogenic induction ($P < 0.05$) (Fig. 6A-B). Matrix mineralization was significantly decreased with miR-195-5p mimics transfection on the 14th day of osteogenic induction. However, the decreased matrix mineralization was rescued by the overexpression of Wnt3a (Fig.

6C), which indicated that miR-195-5p negatively regulated CORM-3-induced osteogenic differentiation of rat BMSCs through directly targeting Wnt3a.

Discussion

Bone defects caused by trauma, tumor and inflammation disease seriously reduces the life quality of patients. Stem cell-based engineering has emerged as a promising and effective approach of bone regeneration (Luby *et al.* , 2019; Polymeri*et al.* , 2016). BMSCs are presently regarded as the gold standard cell source for bone tissue engineering, due to their self-renewal and multipotential differentiation capacity (Dai *et al.* , 2018; Fu*et al.* , 2019; Jing *et al.* , 2019). Multiple biological materials and growth factors have been implemented for osteoinduction of BMSCs (Um *et al.* , 2018; Zhang *et al.* , 2016). CORMs, a novel group of compounds that are carriers of CO (Motterlini *et al.* , 2002), have exhibited such potent effects as anti-inflammatory (Lee *et al.* , 2018), anti-apoptotic (Ulbrich *et al.* , 2016), neuroprotective (Ulbrich *et al.* , 2017), vascular function improvement (Foresti *et al.* , 2004; Motterlini *et al.* , 2002) and so on. Based on our previous study, CORM-3 promotes the osteogenic differentiation of rat BMSCs (Li *et al.* , 2018). However, the mechanism underlying was unknown.

miRNAs, about 22 nucleotides in length, are highly conserved endogenous RNAs. miRNAs can induce translational silence by binding to the 3'UTR of target mRNAs, thus affecting cell proliferation, differentiation, apoptosis, and ontogeny (Jackson *et al.* , 2007; O'Brien *et al.* , 2018; Wang *et al.* , 2019). Several studies have shown that miRNAs play an important role in the osteogenic differentiation of BMSCs (Huang *et al.* , 2019; Li, 2018; Li *et al.* , 2019; Wang*et al.* , 2019). Recently, researchers have uncovered CORMs mechanism associated with miRNAs (Uchiyama *et al.* , 2010). CORM-2 prevents TNF-α-induced endothelial nitric oxide synthase downregulation by inhibiting miR-155-5p biogenesis (Choi *et al.* , 2017). CORM-3 improves structural and functional cardiac recovery after myocardial injury via decreasing miR-206 expression (Segersvard *et al.* , 2018). However, little is known about CORMs and miR-195-5p. In the present study, we first confirmed the down-regulation of miR-195-5p in CORM-3-induced osteogenic differentiation of rat BMSCs, based on the previous gene sequencing result. Our results suggested that miR-195-5p might be involved in the CORM-3-induced osteogenic differentiation of rat BMSCs.

Runx2 is an osteogenesis specific transcription factor and plays an important role in osteogenic differentiation of BMSCs (Komori *et al.* , 1997; Xu *et al.* , 2015). As a marker for early osteogenic differentiation, it can promote the expressions of a number of downstream genes associated with osteogenic differentiation (Deng*et al.* , 2013). OPN is a multifunctional protein mainly associated with bone formation. OPN is secreted by osteoblast, and is a characteristic phenotypic marker of osteoblast. It is stored in bone matrix, and effects the matrix mineralization (De Fusco *et al.* , 2017; Singh *et al.* , 2018). In the present study, up-regulation of miR-195-5p level suppressed the CORM-3-induced osteogenic differentiation of rat BMSCs *in vitro* , evidenced by the results of decreased mRNA and protein expressions of Runx2 and OPN, and matrix mineralization demonstrated. Meanwhile, down-regulation of miR-195-5p level enhanced the expression of osteogenic related factors during the CORM-3-induced osteogenic differentiation. These results suggested that miR-195-5p might be a regulator of CORM-3-induced osteogenic differentiation. However, from our experiment, the matrix mineralization was not enhanced significantly in miR-195-5p-deficient cells. This discrepancy might be due to the consideration as following. First, the mechanism underlying CORM-3-induced osteogenic differentiation is complex. Though the alizarin red staining of the mimics group was significantly decreased than that in the CORM-3 group, the scattered spots of alizarin red staining were still seen, which was significantly enhanced than that in the control group. It suggested that there might have other mechanism involved in the CORM-3-induced osteogenic differentiation of rat BMSCs. Secondly, miRNAs are complex network structure. Down-regulation of miR-195-5p via inhibitor transfection*in vitro* might affect the levels of other miRNAs, which led to compensatory converse effect on osteogenic differentiation.

For further research, we first predicted the potential target gene Wnt3a of miR-195-5p by bioinformatics software. In the following experiment, up-regulation of miR-195-5p level decreased the protein expression of Wnt3a in cells. Conversely, the protein expression of Wnt3a was enhanced in miR-195-5p-deficient cells. The data of the regulatory effect of miR-195-5p on Wnt3a, together with the results from the luciferase reporter

assay, demonstrated that miR-195-5p directly targeted Wnt3a.

The Wnt signaling pathway plays an important role in bone development, and it is strongly implicated in skeletal tissue regeneration and repair (Houschyar *et al.* , 2018; Xu*et al.* , 2014). Activating of the Wnt signaling pathway promotes the osteogenic differentiation of BMSCs *in vitro* (Chen *et al.* , 2019; Zhu *et al.* , 2020). Wnt signaling pathway is involved in the process of promoting fracture healing of rats with nonunion*in vivo* (Sun *et al.* , 2019). Wnts now comprise a family of secreted glycoproteins, in which Wnt3a is included (Nusse *et al.* , 2012; Willert *et al.* , 2012). The Wnt ligands bind receptors on the surface of recipient cells to activate the Wnt pathway. During the fracture repair process, the expressions of many Wnt ligands and receptors are upregulated (Xu *et al.* , 2014). Some miRNAs specifically interact with Wnt ligands, leading to consequent regulation on osteogenesis (Long *et al.* , 2017; Peng *et al.* , 2016). Upregulation of miR-16-2* blocks the Wnt signal pathway by directly targeting Wnt5a. miR-16-2* interferes with Wnt5a to regulate osteogenic differentiation of human BMSCs (Duan *et al.* , 2018). miR-196a promotes osteogenic differentiation of adipose stem cells via regulating Wnt/β-catenin pathway (Ai *et al.* , 2019). Therefore, we hypothesized the involvement of Wnt3a in osteogenesis. As shown in present study, the protein expression of wnt3a was enhanced during the CORM-3-induced osteogenic differentiation of rat BMSCs. Morever, overexpression of Wnt3a enhanced CORM-3-induced osteogenic differentiation, with the increased mRNA and protein expressions of Runx2 and OPN, and matrix mineralization. In contrast, the CORM-3-induced osteogenic differentiation was suppressed in the Wnt3a-deficient cells. These results suggested that Wnt3a promoted the CORM-3-induced osteogenic differentiation of rat BMSCs.

To further verify the regulation effect of miR-195-5p, rescue experiments were conducted by miR-195-5p mimics and pcDNA3.1-Wnt3a co-transfection. The data showed that the mRNA and protein expressions of Runx2 and OPN, and matrix mineralization were decreased in cells transfected with miR-195-5p mimics. However, the decreased results were rescued by miR-195-5p mimics and pcDNA3.1-Wnt3a co-transfection. The Wnt3a was presented as an obvious link of miR-195-5p to osteogenic differentiation of rat BMSCs.

CO, which is a gasotransmitter, displays many physiological roles in several organs and tissues. CO administration has therapeutic potential in many diseases (Gullotta *et al.* , 2012; Mitchell *et al.* , 2010; Segersvard *et al.* , 2018). CO has already been evaluated in phase I clinical testing and the feasibility and anti-inflammatory effects of low-dose CO inhalation in patients with chronic obstructive pulmonary disease have been demonstrated (Bathoorn *et al.* , 2007). However, the toxicity and limitation of CO gas prevented the CO application. CORMs, capable of liberating controlled quantities of CO, have been a valid alternative (Motterlini *et al.* , 2003). CORMs have shown a variety of pharmacological activities, with many reports on the biological applications of CORMs in inflammatory, vascular disease, organ transplantation and cancer (Adach *et al.* , 2019; Forest*et al.* , 2008; Gullotta *et al.* , 2012; Motterlini *et al.* , 2002). CORMs, as a new type of drugs, have therefore tremendous therapeutic potential, and may become a new class of therapeutics against various diseases (Ji *et al.* , 2016).

In summary, the present study indicates that miR-195-5p negatively regulates CORM-3-induced osteogenic differentiation of rat BMSCs by targeting Wnt3a. Our results provide new molecule mechanism for CORMs and theoretical basis for bone tissue engineering.

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Author contributions

J.L., H.C. and H.S. conceived and designed the experiments; J.L., Q.H., J.S., T.L. and M.H. performed the experiments; J.L., T.L. and L.W. analysed the data and drafted relevant text; J.L., Q.H., H.C. and H.S. wrote the manuscript. H.S. sourced funding. All authors have read and approved the final version of this

manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figure legends:

Figure 1. Identification of rat BMSCs and cell viability assay.(A) P3 rat BMSCs morphology was assessed by the phase-contrast microscopy. (B) Effects of CORM-3 (100, 200 or 400 μM) on rat BMSCs viability. (C and D) Identification of osteogenic differentiation. Alizarin red staining of rat BMSCs cultured in the osteogenic (C) or control (D) medium for 21 days. (E and F) Adipogenic differentiation identification. Oil red O staining of rat BMSCs cultured in the adipogenic (E) or control (F) medium for 21 days. (G and H) Clonogenesis experiment. Rat BMSCs were seeded at a density of 3 cells/cm^2 and cultured in control

medium. After 10 days, crystal violet staining of rat BMSCs was observed by visual (G) and the phase-contrast microscopy (H). Magnification, x100; scale bar, 100 μm. The experiment was repeated in triplicate. Data were presented as the mean ± standard deviation (n=3). * $P < 0.05$ as indicated.

Figure 2. Expressions of miR-195-5p and Wnt3a during osteogenic differentiation of rat BMSCs. Rat BMSCs in the CORM-3 or osteogenic group were cultured in the osteogenic medium with or without 200 μM CORM-3, respectively. Rat BMSCs in the control group were cultured in the control medium. (A) RT-qPCR analysis of miR-195-5p on 24 hours, normalized to U6. (B) Representative western blot images of three independent experiments for Wnt3a protein expression at 24, 48 and 72 hours and quantitative results of western blot images, using ImageJ software, normalized to Tubulin. The experiment was repeated in triplicate. Data were presented as the mean ± standard deviation (n=3). as indicated.

Figure 3. Effects of miR-195-5p on the CORM-3-induced osteogenic differentiation of rat BMSCs. (A) The rat BMSCs were transfected with miR-195-5p mimics or mimics NC, miR-195-5p inhibitor or inhibitor NC. After 48 hours, the expressions of miR-195-5p were determined by RT-qPCR, normalized to U6. (B and C) The rat BMSCs were transfected with miR-195-5p mimics or mimics NC (B), miR-195-5p inhibitor or inhibitor NC (C) for 24 hours and then cultured in the osteogenic medium containing 200 μM CORM-3. Meanwhile, cells in the osteogenic, CORM-3 or control group were cultured in the osteogenic medium, osteogenic medium containing 200 μM CORM-3 or control medium respectively. After 3 and 7 days, the mRNA expressions of Runx2 and OPN were determined by RT-qPCR, normalized to β-actin. (D and E) The rat BMSCs were cultured as (B and C) described above. After 3 and 7 days, the protein expressions of Runx2 and OPN were determined by Western blot, then analysed using ImageJ software, normalized to GAPDH. (F) The rat BMSCs were cultured in different mediums as described above. After 14 days, the mineralization was determined by alizarin red staining and semi quantitative analysis. The experiment was repeated in triplicate. Data were presented as the mean ± standard deviation (n=3). ^ $P < 0.05$ vs. control; * $P < 0.05$ as indicated.

Figure 4. miR-195-5p directly targets Wnt3a. (A) The design of luciferase reporters with Wnt3a 3'UTR-wt or Wnt3a 3'UTR-mut. (B and C) Western blot images and analysis for Wnt3a protein expression in rat BMSCs after 48 hours transfection with miR-195-5p mimics or mimics NC (B), miR-195-5p inhibitor or inhibitor NC (C), using ImageJ software, normalized to Tubulin. (D) Effect of miR-195-5p mimics on luciferase activity in 293T cells transfected with either the 3'UTR-wt reporter or the 3'UTR-mut reporter for Wnt3a. The experiment was repeated in triplicate. Data were presented as the mean +- standard deviation (n=3). as indicated.

Figure 5. Effects of Wnt3a on the CORM-3-induced osteogenic differentiation of rat BMSCs. (A) Western blot images and analysis for Wnt3a protein expression in rat BMSCs after 48 hours transfection with pcDNA3.1-Wnt3a, Wnt3a siRNA or their corresponding controls. (B and C) The rat BMSCs were transfected with pcDNA3.1-Wnt3a or NC (B), Wnt3a siRNA or NC (C) for 24 hours and then cultured in the osteogenic medium containing 200 μM CORM-3. Meanwhile, cells in the CORM-3 or control group were cultured in the osteogenic medium containing 200 μM CORM-3 or control medium respectively. After 3 and 7 days, the mRNA expressions of Runx2 and OPN were determined by RT-qPCR, normalized to β-actin. (D and E) The rat BMSCs were cultured as (B and C) described above. After 3 and 7 days, the protein expressions of Runx2 and OPN were determined by Western blot, then analysed using ImageJ software, normalized to GAPDH. (F) The rat BMSCs were cultured in different mediums as described above. After 14 days, the mineralization was determined by alizarin red staining and semi quantitative analysis. The experiment was repeated in triplicate. Data were presented as the mean ± standard deviation (n=3). as indicated.

Figure 6. miR-195-5p affected CORM-3-induced osteogenic differentiation of rat BMSCs by targeting Wnt3a. (A) The rat BMSCs were co-transfected with miR-195-5p mimics and pcDNA3.1-Wnt3a, miR-195-5p mimics and NC for 24 hours and then cultured in the osteogenic medium containing 200 μM CORM-3. Meanwhile, cells in the CORM-3 or control group were cultured in the osteogenic medium containing 200 μM CORM-3 or control medium respectively. After 3 and 7 days, the mRNA expressions of

Runx2 and OPN were determined by RT-qPCR, normalized to β-actin. (B) The rat BMSCs were cultured in different mediums as described above. After 3 and 7 days, the protein expressions of Runx2 and OPN were determined by Western blot, then analysed using ImageJ software, normalized to GAPDH. (C) The rat BMSCs were cultured in different mediums as described above. After 14 days, the mineralization was determined by alizarin red staining and semi quantitative analysis.

The experiment was repeated in triplicate. Data were presented as the mean ± standard deviation (n=3). ˆ $P < 0.05$ vs. control; * $P < 0.05$ as indicated.